CSHL91-02AAA
PG6
EWM/PG/jzg
12/17/92

Attachment to 33/I

PATENT APPLICATION
Docket No. CSHL91-02AAA

A58149-3
CSHL-003-2
copy of USSN 07/991,997

CYCLIN COMPLEX REARRANGEMENT AND USES RELATED THERETO

Description

Related Applications

This application is a continuation-in-part of U.S.S.N. Serial Number 07/963,308 filed October 16, 1992 and entitled "D-Type Cyclin and Uses Related Thereto" which is a continuation-in-part of U.S.S.N. Serial Number 07/888,178 filed May 26, 1992 and entitled "D-Type Cyclin and Uses Related Thereto", which corresponds to and claims priority to Patent Cooperation Treaty Application No. PCT/US92/04146, filed May 18, 1992 and entitled "D-Type Cyclin and Uses Related Thereto", and to U.S.S.N. 07/701,514, filed May 16, 1991 and entitled "D-Type Cyclin and Uses Related Thereto." The teachings of U.S.S.N. 07/963,308, 07/888,178, 07/701,514 and the PCT Application are incorporated herein by reference.

Funding

Work described herein was supported by National Institutes of Health Grant GM39620 and the Howard Hughes Medical Institute. The United States Government has certain rights in the invention.

Background of the Invention

Cyclins are proteins that were discovered due to their intense synthesis following the fertilization of marine invertebrate eggs (Rosenthal, E.T. et al., Cell 20:487-494 (1980)). It was subsequently observed that the abundance of two types of cyclin, A and B, oscillated during the early cleavage divisions due to abrupt proteolytic degradation of the polypeptides at mitosis and thus,

they derived their name (Evans, T. et al., Cell 33:389-396 (1983); Swenson, K.I. et al., Cell 47:867-870 (1986); Standart, N. et al., Dev. Biol. 124:248-258 (1987)).

Active rather than passive involvement of cyclins in regulation of cell division became apparent with the observation that a clam cyclin mRNA could cause activation of frog oocytes and entry of these cells into M phase (Swenson, K.I. et al., Cell 7:867-870 (1986)). Activation of frog oocytes is associated with elaboration of an M phase inducing factor known as MPF (Masui, Y. and C.L. Markert, J. Exp. Zool. 177:129-146 (1971); Smith, L.D. and R.E. Ecker, Dev. Biol. 25:232-247 (1971)). MPF is a protein kinase in which the catalytic subunit is the frog homolog of the cdc2 protein kinase (Dunphy, W.G. et al., Cell 54:423-431 (1988); Gautier, J. et al., Cell 54:433--439 (1988); Arion, D. et al., Cell 55:371-378 (1988)).

Three types of classes of cyclins have been identified to date: B, A and CLN cyclins. The B-type cyclin has been shown to act in mitosis by serving as an integral subunit of the cdc2 protein kinase (Booher, R. and D. Beach, EMBO J. 6:3441-3447 (1987); Draetta, G. et al., Cell 56:829-838 (1989); Labbe, J.C. et al., Cell 57:253-263 (1989); Labbe, J.C. et al., EMBO J. 8:3053-3058 (1989); Meier, L. et al., EMBO J. 8:2275-2282 (1989); Gautier, J. et al., Cell 60:487-494 (1990)). The A-type cyclin also independently associates with the cdc2 kinase, forming an enzyme that appears to act earlier in the division cycle than mitosis (Draetta, G. et al., Cell 56:829-838 (1989); Minshull, J. et al., EMBO J. 9:2865-2875 (1990); Giordano, A. et al., Cell 58:981-990 (1989); Pines, J. and T. Hunter, Nature 346:760-763 (1990)). The functional difference between these two classes of cyclins is not yet fully understood. Cellular and molecular studies of cyclins in invertebrate and vertebrate embryos have been accompanied by genetic studies, particularly in

ascomycete yeasts. In the fission yeast, the cdc13 gene encodes a B-type cyclin that acts in cooperation with cdc2 to regulate entry into mitosis (Booher, R. and D. Beach, EMBO J., 6:3441-3447 (1987); Booher, R. and D. Beach, EMBO J. 7:2321-2327 (1988); Hagan, I. et al., J. Cell Sci. 91:587-595 (1988); Solomon, M., Cell 54:738-740 (1988); Goebl, M. and B. Byers, Cell 54:433-439 (1988); Booher, R.N. et al., Cell 58:485-497 (1989)). Genetic studies in both the budding yeast and fission yeast have revealed that cdc2 (or CDC28 in budding yeast) acts at two independent points in the cell cycle: mitosis and the so-called cell cycle "start" (Hartwell, L.H., J. Mol. Biol., 104:803-817 (1971); Nurse, P. and Y. Bissett, Nature 292:558-560 (1981); Piggot, J.R. et al., Nature 298:391-393 (1982); Reed, S.I. and C. Wittenberg, Proc. Nat. Acad. Sci. USA 87:5697-5701 (1990)). In budding yeast, the start function of the CDC28 protein also requires association of the catalytic subunit of the protein kinase with ancillary proteins that are structurally related to A and B-type cyclins. This third class of cyclin has been called the CLN class, and three genes comprising a partially redundant gene family have been described (Nash, R. et al., EMBO J. 7:4335-4346 (1988); Hadwiger, J.A. et al., Proc. Natl. Acad. Sci. USA 86:6255-6259 (1989); Richardson, H.E. et al., Cell 59:1127-1133 (1989)). The CLN genes are essential for execution of start and in their absence, cells become arrested in the G1 phase of the cell cycle. The CLN1 and CLN2 transcripts oscillate in abundance through the cell cycle, but the CLN3 transcript does not. In addition, the CLN2 protein has been shown to oscillate in parallel with its mRNA (Nash, R. et al., EMBO J. 7:4335-4346 (1988); Cross, F.R., Mol. Cell. Biol. 8:4675-4684 (1988); Richardson, H.E. et al., Cell 59:1127-1133 (1988); Wittenberg, et al., 1990)). Although the precise biochemical properties conferred on

cdc2/CDC28 by association with different cyclins have not been fully elaborated, genetic studies of cyclin mutants clearly establishes that they confer "G1" and "G2" properties on the catalytic subunit (Booher, R. and D. Beach, EMBO J. 6:3441-3447 (1987); Nash, R. et al., EMBO J. 7:4335-4346 (1988); Richardson, H. E. et al., Cell 56:1127-1133 (1989)).

cdc2 and cyclins have been found not only in embryos and yeasts, but also in somatic human cells. The function of the cdc2/cyclin B enzyme appears to be the same in human cells as in other cell types (Riabowol, K. et al., Cell 57:393-401 (1989)). A human A type cyclin has also been found in association with cdc2. No CLN type cyclin has yet been described in mammalian cells. A better understanding of the elements involved in cell cycle regulation and of their interactions would contribute to a better understanding of cell replication and perhaps even alter or control the process.

Summary of the Invention

The present invention relates to uses for the novel class of cyclins, referred to as D-type cyclins, which are of mammalian origin and are a new family of cyclins related to, but distinct from, previously described A, B or CLN type cyclins. In particular, it relates to human cyclins, encoded by genes shown to be able to replace a CLN-type gene essential for cell cycle start in yeast, which complement a deficiency of a protein essential for cell cycle start and which, on the basis of protein structure, are on a different branch of the evolutionary tree from A, B or CLN type cyclins. D-type cyclins have been shown to associate, in eukaryotic cells, particularly human cells, with multiple cyclin dependent kinases. They have also been shown to co-precipitate with three polypeptides: a cyclin-dependent kinase, a well characterized DNA replica-

tion and repair factor (i.e., proliferating cell nuclear antigen or PCNA) and a polypeptide of 21 kDa apparent molecular weight. Results suggest that D-type cyclin, CDK, PCNA and p21 exist in a quaternary complex, that many combinatorial variations of the components (e.g., cyclin D1 or D3 and CDK2, CDK4 and CDK5) assemble <u>in vivo</u> and that each of the quaternary complexes may have a subtly different role in the cell cycle or in different cell types. In addition, it has been discovered that cellular transformation by DNA tumor viruses, such as SV40, is associated with selective subunit rearrangement of the cyclin D complexes. The association between cyclin D, PCNA, CDKs (including CDK2, CDK4 and CDK5) and p21 is disrupted by introduction of a DNA tumor virus or its oncogenic gene product into mammalian cells. Specifically, as described herein, it has been shown that the association between cyclin D and PCNA, CDKs (CDK2, CDK4, and CDK5) and p21 is disrupted upon introduction of SV40 tumor virus or its oncogenic gene product large T antigen into human diploid cells (as exemplified by normal human diploid fibroblasts). After disassociating from cyclin D and p21, CDK4 becomes associated with a novel polypeptide of 16 kDa (p16). Similarly, cyclin A complexes also undergo subunit rearrangement. After SV40 transformation, p21 association with cyclin A is decreased or completely disassociated. Cyclin A then appears in a complex with a 19 kDA polypeptide (p19).

Thus, it is now known that p21 is associated with cyclin kinases only in normal, untransformed cells, and p16, p19 and potentially other related proteins appear to the cell cycle regulator present in transformed cells. This knowledge serves as the basis for a variety of approaches to modulating cell division by altering the activity (directly or indirectly) of cyclins. It offers specificity in modulating cell division (i.e., the ability

to selectively alter cell division in particular cell types or at a particular point in the cycle) because of the specificity of expression of cyclins in cells and the number of possible combinations of the components of the quaternary complex which appear to be formed by cyclins, CDK, PCNA and p21. In a particular embodiment, it offers a means by which cell division can be non-specifically altered by interfering with a common component of the quaternary complex of which D-type cyclin or A-type cyclin is a constituent, such as by interfering with PCNA.

For example, in one embodiment of a therapeutic method of the present invention, formation of the quaternary complex described above is prevented or enhanced or the activity of a complex member is altered as an approach to altering cell division. Here, agents which act indirectly or directly to prevent or enhance complex formation or to alter a constituent's activity can be used. For example, as described above, catalytic activity can be inhibited by preventing activation of the protein kinase. Alternatively, PCNA inhibitors can be introduced into cells in which cell cycle start is to be inhibited, resulting in inhibition of cell division. PCNA inhibitors can act indirectly (e.g., to reduce production of PCNA by interfering with transcription or translation) or directly (e.g., to bind PCNA and prevent it from joining with other complex members). Inhibitors of p21 can also be introduced into cells and interfere, indirectly or directly, with p21 function and/or binding to the complex members. Protein-protein interactions (between or among complex components) can also be altered (reduced or enhanced) to have the desired effect on the cell cycle (to reduce or increase cell division). Agents which block such protein-protein interactions can be used. These include low molecular weight inhibitors, agents which bind to complex components (e.g., antibodies) and agents which degrade or

otherwise destroy a component's ability to form a complex with the other proteins. If enhanced quaternary complex formation is desired, agents which increase the ability of complex members to interact and bind (e.g., agents which change the configuration of a complex component so that it is more available for protein-protein interactions necessary for complex formation can be introduced into cells). Enhanced complex formation can also be brought about by increasing in cells the number, activity or availability of the limiting member(s) of the quaternary complex, thus enhancing the rate at which it is formed and its availability to act.

In addition, the subject invention pertains to methods of diagnosing transformation of a cell. Reagents, such as monoclonal antibodies, can be developed that recognize the interactions between the CDKs, cyclins, PCNA and the low molecular weight polypeptides (e.g., p21, p19 and p16). For example, an antibody which recognizes the interaction between p16 and CDK4 can be used to detect or diagnose transformation of many cell types. Alternatively, agents such as antibodies which recognize CDC2, CDK2, cyclin A or cyclin D, can be used to identify the subunit composition of the cyclin complexes and thus the state of transformation of the cell.

The subject invention also relates to agents (e.g., oligonucleotides, antibodies, peptides) useful in the isolation, diagnostic or therapeutic methods described.

Brief Description of the Drawings

Figure 1 is a schematic representation of potential combinational interactions of D-type cyclins, cyclin-dependent kinases, PCNA and p21.

Figure 1A is a summary of established pair-wise protein-protein interactions, in which each arrow indicates a demonstrated co-precipitation between two proteins.

Figure 1B is a schematic representation of the proposed quaternary complex between D-type cyclins, CDKs, p21 and PCNA.

Figure 2 shows that cellular transformation with DNA tumor virus SV40 is associated with subunits rearrangement of cell cycle complexes.

Figure 3 shows subunit rearrangements of cell cycle complexes in two different pair cell lines.

Figure 4 shows that cellular transformation by DNA tumor virus SV40 is associated with rearrangement of PCNA subunit of cell cycle complexes.

Figure 5 shows that cellular transformation by DNA tumor virus SV40 is associated with rearrangement of CDK4 subunit of cell cycle complexes.

Figure 6 shows that cellular transformation by other DNA tumor viruses is also associated with subunits rearrangement of cell cycle complexes.

Detailed Description of the Invention

Applicant has determined that in eukaryotic cells, specifically human cells, D-type cyclin associates with multiple catalytic subunits (cyclin dependent kinases or CDK). Applicant has also shown that D-type cyclin and CDK co-precipitate with two additional polypeptides: a well characterized DNA replication and repair factor (i.e., proliferating cell nuclear antigen or PCNA) and a polypeptide of 21 kDa apparent molecular weight. Results suggest

that D cyclin, CDK, PCNA and p21 exist in a quaternary complex, that many combinatorial variations of the components (e.g., cyclin D1 or D3 and CDK2, CDK4 and CDK5) assemble in vivo and that each of the resulting quaternary complexes may have a subtly different role in the cell cycle or in different cell types.

Applicant's work, thus, provides the first biochemical indication of a possible function of D-type cyclins (i.e., as modulators of PCNA function) and, for the first time, provides evidence of the role of the quaternary complex in cellular transformation. The following is a description of the discovery that D-type cyclin is associated with three additional polypeptides (CDK, PCNA and p21) in what appears to be a quaternary complex in which many combinatorial variations are possible, resulting in a variety of resulting complexes which may play different roles in the cell cycle or in different cell types.

As described below and in Example 1, immunological procedures have been used to establish that D-type cyclins associate, in eukaryotic cells, with a variety of potential catalytic subunits (e.g., CDKs, such as CDK2, CDK4 and CDK5). In addition, these procedures have shown that the D-type cyclin and CDK associate with the replication factor PCNA and a polypeptide of 21 kDa apparent molecular weight. The various pair-wise interactions possible are summarized in Figure 1A.

Human cyclin D1 has been associated with a wide variety of proliferative diseases, but its biochemical role is unknown. As described herein, in human diploid cells, specifically human diploid fibroblasts, cyclin D1 is complexed with many other cellular proteins. Among them are protein kinase catalytic subunits CDK2, CDK4 (previously called PSK-J3), and CDK5 (also called PSSALRE). In addition, polypeptides of 21 kDa and 36 kDa are identified in association with cyclin D1. As des-

cribed in Example 1, it has been shown that the 36 kDa protein is the proliferating cell nuclear antigen, PCNA. PCNA has been described as an essential accessory factor to the delta polymerase, which is required for leading-strand DNA replication and DNA repair. Cyclin D3 also associates with multiple protein kinases, p21 and PCNA, as shown herein. It is proposed that there exists a quaternary complex of D cyclin CDK, PCNA and p21 and that many combinatorial variations (cyclin D1, D3, CDK2, 4 and 5) may assemble *in vivo*. These findings link a human putative G1 cyclin that is associated with oncogenesis with a well characterized DNA replication and repair factor.

Investigation of Proteins that Associate with Cyclin D

To identify proteins that specifically associate with cyclin D, anti-cyclin D1 immunoprecipitates of [^{35}S] methionine-labelled WI38 human diploid fibroblasts lysates were examined (see Example 1, Experimental Procedures). WI38 cells were initially chosen for this study because they are a relatively normal cell line that expresses reasonably high levels of cyclin D1 and a low level of cyclin D3 mRNA (Won *et al.*, *Proc. Natl. Acad. Sci.* (1992). Human 293 transformed primary embryonal kidney cells were used as controls because they express all three D cyclin mRNAs and proteins at extremely low levels (Xiong, *et al.*, *Cell* *65*:691-699 (1991); data not shown). W138 cells express a readily detectable 35 kDa polypeptide that can be immunoprecipitated by the anti-cyclin D1 antiserum. The identity of the 35 kDa protein as cyclin D1 was confirmed by comparison of an immunoprecipitate of the same W138 cell lysate with pre-immune serum, and with a similar precipitation of 293 cell lysate with the same anti-cyclin D1 antiserum (data not shown). Because of the existence of three closely related cyclin D genes in human cells, and weak cross-reactivity of the anti-cyclin D1 antibody

to other cyclin D proteins, the identity of the 35 kDa band was further investigated by partial proteolytic mapping. S. aureus V8 partial proteolysis of the 35 kDa band revealed the same pattern as that of similar cleaved cyclin D1 synthesized in vitro, but not as that of cyclin D2 or D3.

In addition to the intense 35 kDa band corresponding to cyclin D1, three other major bands, p33 and p21 and one minor band, p31, appeared specifically in the anti-cyclin D1 precipitates (data not shown). These polypeptides are absent from precipitates of W138 cell lysate using pre-immune serum or precipitates of 293 cell lysates with the same anti-cyclin D1 antibody (data not shown). The possibility that any of these four bands, in particular p31 and p33, might be cyclin D2 or D3 was ruled out by comparing their partial V8 proteolysis patterns with those of in vitro translated D2 and D3. Precipitation of these polypeptides with anti-cyclin D1 serum is also not likely due to the presence of cross-reactive epitopes in any of these proteins, since they were not detected following immunoprecipitation coupled with Western blotting using the same antibody. Experiments to identify the cyclin D1-associated proteins are described below.

CKD5 Associates with D-Type Cyclins

It has been previously reported that murine macrophages cyclin D1/cyl1 associates with a polypeptide that cross-reacts with an antibody to full-length p34cdc2 of Schizosaccharomyces pombe (G8), but not with an antibody prepared against the C-terminus of human p34cdc2 (Draetta et al., Cell 50:319-325 (1987); Draetta and Beach, Cell 54:17-26. (1988); Matsushime et al.,Cell 65:701-713 (1991). Essentially identical results were obtained in human W138 cells, suggesting that cyclin D1 associates with a relative of human CDC2.

The G8 antibody was used to screen human cDNA expression libraries (see Example 1, Experimental Procedures), in order to isolate putative D-type cyclin-associated kinases. Thirty four G8-positive cDNA clones were identified from a HeLa cell cDNA library. Among these, 17 clones encoded CDC2 and another 14 encoded for CDK2. One of the remaining clones encodes an ORF of 292 amino acid residues with a predicted molecular weight of 33,283 daltons. This clone is designated CDK5, since it shares extensive amino acid identity to the known cyclin-dependent kinases (CDKs), including *S. pombe* CDC2 (53.4%), *S. cerevisiae* CDC28 (55.9%), human CDC2 (56.8%), and human CDK2 (60.3%), and associates with human D-type cyclins (see below). CDK5 has an inferred amino-acid sequence that is almost identical to a putative protein kinase which was recently identified using polymerase chain reaction (PCR) with primers that are conserved among cdc2 genes (Meyerson *et al.*, *EMBO J.* 11:2909-2917 (1992)). CDK5 encodes a sequence of DLKKYFD at amino acid sequence 86 to 92 and the protein referred to as PSSALRE (Meyerson *et al.*, *EMBO J.* 11:2090-2917 (1992)) contains DLK-NFD at the corresponding region. It is not known whether these two polypeptides are derived from two genes, spliced differently, or whether the discrepancy might have arisen from a cloning or sequencing artifact. In the corresponding region, human CDC2 has the sequence of DLKKYLD and CDK2 has DLKKFMD.

To determine whether CDK5 associates with D cyclins, an antiserum was raised against a peptide corresponding to the unique carboxy-terminal region of CDK5 (see Example 1, Experimental Procedures). This serum does not cross react with human CDC2, CDK2, or CDK4. Immunoprecipitation or Western-blotting following immunoprecipitation showed that this antiserum detected a polypeptide with a M_r 31 kDa (p31) from cell lysate, which co-migrated with CDK5 poly-

peptide synthesized in vitro and was effectively competed away by the CDK5 antigenic peptide (data not shown). The identity of the 31 kDa protein precipitated by the anti-CDK5 antibody was further confirmed to be CDK5 by comparing the partial V8 proteolytic mapping of p31 with in vitro translated CDK5.

Immunoprecipitation of cell lysates of ^{35}S-methionine labeled W138 cells using the anti-CDK5 antiserum revealed several polypeptides, in addition to p31^{CDK5}. Among these, polypeptides of 36 kDa (p36), p35 kDa (p35), 33 kDa (p33) and 21 kDa (p21) were most prominent and specifically coprecipitated by the anti-CDK5 antiserum. All four polypeptides were absent from precipitates with the pre-immune serum or in the presence of excess amount of the CDK5 carboxy-terminal peptide (data not shown).

The electrophoretic mobilities of p35 and p33 were found to be the same as that of <u>in vitro</u> translated human cyclin D1 and D3, respectively. To directly test the possibility that the CDK5-associated p35 might correspond to cyclin D1, CDK5 immunoprecipitates were blotted with anti-cyclin D1 antisera. A 35 kDa polypeptide, which co-migrated with p35$^{cyclin\ D1}$, was detected by the anti-cyclin D1 antiserum. Reciprocal blotting of anti-cyclin D1 immunocomplexes by the CDK5 antiserum also revealed the presence of a 31 kDa polypeptide which had the same mobility as p31^{CDK5}. Similarly, CDK5 has also been detected in anti-cyclin D3 immunoprecipitates. These data suggest that the CDK5-associated p35 is cyclin D1 and CDK5-associated p33 is cyclin D3.

To seek conclusive evidence of the identity of the CDK5-associated p35 and p33 proteins, partial proteolytic mapping was employed (Cleveland <u>et al</u>., <u>J. Biol. Chem.</u> <u>252</u>:1102-1106 (1977)). ^{35}S-labelled p35 purified from anti-CDK5 immunoprecipitates was subjected to partial <u>S. aureus</u> V8 protease digestion and compared with similarly

treated human p35^{cyclinD1} obtained either from _in vitro_ translation or from an anti-cyclin D1 immunoprecipitation. The V8 proteolytic pattern of p35 from anti-CDK5 immuno-precipitates was identical to that of cyclin D1, but distinct from that of cyclin D3. Similar experiments were also performed to confirm the identity of p33. The partial proteolytic pattern of the CDK5-associated p33 is identical to that of an vitro translated human cyclin D3, but not D1. Conversely, it has also been determined that the partial V8 digestion pattern of the cyclin D1-associated p31 is identical to CDK5 obtained either from in vitro translation or anti-CDK5 immunoprecipitation (data not shown).

CDK2 Associates with Cyclin D

The apparent molecular weight of the cyclin D1-associated p33 and also the cross reactivity of p33^{CDK2} with the G8 antibody suggests the possibility that p33 might be CDK2. To test this, anti-CDK2 precipitate of a [^{35}S] methionine-labelled WI38 cell lysate was compared with an anti-cyclin D1 precipitate. As expected, the anti-C terminal CDK2 serum precipitated a 33 kDA protein which was confirmed to be p33^{CDK2} by comparing the partial _S. aureus_ V8 proteolysis pattern of the 33 kDa band with that of _in vitro_ translated CDK2. p33^{CDK2} comigrated with the p33 present in the anti-cyclin D1 precipitate. Reciprocally, anti-CDK2 antiserum also precipitated a 35 kDa protein which comigrated with cyclin D1 (data not shown).

To seek further evidence for the existence of a possible association between CDK2 and cyclin D1, a WI38 cell lysate was immunoprecipitated with anti-cyclin D1, separated on SDS-PAGE and immunoblotted with anti-CDK2 antiserum. The anti-CDK2 antibody was raised against a carboxy-terminal peptide (Pagano _et al._, _EMBO J._ _11_:961-971 (1992)) and its specificity was checked by immunoblotting bacterially expressed human CDC2, CDK2, CDK3,

CDK4 and CDK5. Only CDK2, and not the other four CDK proteins, was recognized by this antibody. CDK2 protein was detected in the precipitate with anti-CDK2 and anti-cyclin D1, but not in that with pre-immune serum nor with anti-CDK2 pre-incubated with competing antigenic peptides. In a reciprocal Western blot experiment, cell lysate was immunoprecipitated with anti-CDK2 and blotted with anti-cyclin D1. Cyclin D1 was detected in the anti-cyclin D1 and anti-CDK2 immunoprecipitates, but not in precipitates with either preimmune serum or anti-CDK2 antiserum pre-incubated with a competing CDK2 peptide.

To test whether CDK2 also associates with cyclin D3, immunoprecipitates using antiserum to the C-terminal peptide of human cyclin D3 (see Example 1, Experimental Procedures) were blotted with anti-CDK2 antiserum. CDK2 was weakly detected in the anti-cyclin D3 precipitate, but not in the control precipitate with anti-cyclin D3 antiserum pre-incubated with a competing antigen peptide.

Finally, to further confirm the association between CDK2 and cyclin D, partial proteolytic mapping experiments were conducted. Initially, attempts were made to proteolytically map the cyclin D1-associated p33 to compare it with CDK2. However, because of the comigration of CDK2 with yet another predominant protein kinase in the anti-cyclin D1 precipitates, a different proteolytic pattern was obtained. Therefore, the converse experiment was performed. The 35 kDa band in anti-CDK2 immunoprecipitates was excised from SDS-polyacrylamide gel, partially digested with V8 protease and electrophoretically separated and compared with V8 digested $p35^{cyclin\ D1}$ derived either from in vitro translation or from an anti-cyclin D1 immunoprecipitation. The pattern of proteolytic cleavage was the same in each case.

pSK-J3/CDK4 is the Predominant p33 Protein Associated with Cyclin D1

The difference in the proteolytic pattern of cyclin D1-associated p33 from that of CDK2 suggested that the majority of D1-associated p33 corresponds to a protein other than CDK2. During attempts to identify this protein, it was suggested to us by Dr. Charles Sherr (St. Jude Children's Research Hospital, Tennessee) that a protein kinase called PSK-J3, originally identified in a screen with mixed oligonucleotide probes derived from conserved regions of serine/threonine kinases (Hanks, S.K., Proc. Natl. Acad. Sci. USA 84:388-392 (1987)), may have cyclin D binding properties. The predicted molecular mass of PSK-J3 is 34 kDa, close to that of p33. Because of its association with D cyclins, as demonstrated below, PSK-J3 is referred to hereinafter as CDK4. *In vitro* translated CDK4, and that precipitated from a cell lysate with anti-CDK4 serum, showed the same electrophoretic mobility as CDK2 and the D1-associated p33 (data not shown). The identify of CDK4 precipitated by the anti-CDK4 antiserum was confirmed by comparing its partial V8 mapping pattern to that of *in vitro* translated CDK4.

Immunoprecipitation-Western blotting experiments were carried out to directly test whether the cyclin D1-associated p33 is CDK4. An anti-CDK4 serum reacted with a 33 kDa protein present in anti-cyclin D1 immunoprecipitates that has the same mobility as the CDK4 precipitated by anti-CDK4, but did not react with precipitates of either CDK2 or CDK5. Reciprocally, the anti-CDK4 antiserum also precipitated a 35 kDa protein detected by anti-cyclin D1 antibody. To further confirm the identity of the cyclin D1-associated p33, the partial V8 digestion pattern of p33 was compared to that of immunoprecipitated CDK4 and CDK2. The cyclin D1-associated p33 displayed a very similar pattern to that of CDK4, but was quite dissimilar to that

of CDK2. This result indicates that CDK4 is considerably more abundant (at least as crudely assayed by methionine labelling) than CDK2 in anti-cyclin D1 precipitates of WI38 cells. Similarly, a 33kDa polypeptide (p33) seen in anti-CDK4 immunoprecipitate has been identified to be cyclin D3 by partial V8 peptide mapping.

<u>Association of p21 with Cyclin D1 and CDK2</u>

In [^{35}S] methionine-labelled WI38 lysate precipitated with anti-cyclin D1 serum, a 21 kDa protein (p21) appeared to associate specifically with cyclin D1 (data not shown). p21 was not present in the precipitates with pre-immune serum, nor in the anti-cyclin D1 precipitate derived from 293 cells which contains undetectable levels of cyclin D1 (data not shown). Specific association of p21 with cyclin D1 was further supported by the presence of a comigrating 21 kDA protein in immunoprecipitates with sera against CDK2, CDK4 and CDK5 (data not shown). If anti-CDK2 antiserum was pre-blocked with a competing CDK2 peptide, the p21 band, and also $p33^{CDK2}$ and $p35^{cyclin\ D1}$ were not seen. Similarly, p21 was also absent from anti-CDK5 immunoprecipitates if the antiserum was pre-incubated with the CDK5 carboxy-terminal antigen peptide (data not shown). p21 was not recognized in Western blots by any of the anti-CDK or anti-cyclin D antibodies used in this study. Furthermore, although the total immunoprecipitable CDK2 in 293 cells is similar to that in WI38 cells, the p21 band was not present in the CDK2 immunoprecipitates from 293 cell lysates. This finding suggests that the association of CDK2 and p21 is dependent on cyclin D.

To determine whether the p21 from cyclin D1 immunoprecipitates and CDK2 immunoprecipitates correspond to the same polypeptide, the partial V8 prot olytic pattern of the p21 purified from each source were compared. They are indeed the same. The p21 precipitated by anti-CDK5 anti-

serum was also found to be the same as cyclin D1-associated p21. The p21 in the anti-CDK4 immunoprecipitation was also proteolytically mapped (data not shown). It gave an identical pattern to the cyclin D1-associated p21. p21 does not correspond to the human max protein or $p21^{ras}$, as its electrophoretic mobility is faster than that of either and it was not recognized by an anti-human ras antibody on Western blots. The molecular identity of p21 is presently unknown.

Cyclin D1-Associated p36 is PCNA

Cyclin D1 precipitates of WI38 cells show associated polypeptides of 21 kDa, 31 kDa and 33 kDa and also a prominent protein of 36 kDa (data not shown). p36 was not detected in control precipitates, using either pre-immune serum or in 293 lysates. A 36 kDa protein, in a lower abundance was also detected in CDK2, CDK4 and CDK5 immunoprecipitates, but not in the precipitates with antiserum pre-incubated with competing peptides (data not shown).

While attempting to establish the identity of the p36, four observations suggested the possibility that it might be the human proliferating nuclear antigen, PCNA. First, in an asynchronous population of proliferating WI38 cells, cyclin D1 was predominantly a nuclear protein (data not shown), although the distribution is not identical to the speckled pattern of PCNA (Bravo, R. and H. MacDonald-Bravo, EMBO J., 4:655-661 (1985); Madsen, P. and J.E. Celis, FEBS Lett., 193:5-11 (1985). Second, while the level of cyclin D1 is relatively constant in mitogenically activated WI38 cells, the p36 in [^{35}S] methionine-labelled cyclin D1 immunoprecipitates was low in quiescent cells and increased at 10-14 hours after stimulation. Ten to fourteen hours after serum stimulation, many WI38 cells are in the late G1, a time which coincides with the onset of PCNA synthesis in serum-stimulated 3T3 fibroblasts (Bravo, R. and H. MacDonald-Bravo, EMBO J., 3:3177-3181

(1984); Celis, J.E. and A. Celis, Proc. Natl. Acad. Sci., USA, 82:3262-3268 (1985); Madsen P. and J.E. Celis, FEBS Lett., 193:5-11 (1985). Third, the apparent molecular weight of p36 is similar to that of PCNA. Finally, anti-PCNA antibody precipitated a 35 kDa polypeptide whose electrophoretic mobility is similar to that of $p35^{cyclin\ D1}$ (data not shown). The identify of the p36 precipitated by the anti-PCNA antibody has been confirmed as PCNA by comparing its V8 peptide map to that of in vitro translated PCNA.

Immunoprecipitation-Western blot experiments were carried out to test directly the possibility that p36 is PCNA. PCNA was readily detected in anti-cyclin D1, cyclin D3, CDK2 and CDK5 immunoprecipitates, but not in the respective control precipitates. In a reciprocal experiment, cyclin D1 and CDK2 were also detected in anti-PCNA immunoprecipitates. It has not been possible to convincingly detect cyclin D3 or CDK5 in PCNA precipitates, possibly due to the low abundance of both proteins in WI38 cells and the relatively poor sensitivity of the D3 and CDK5 antisera in Western blots.

To further assess the similarity between the PCNA and the p36 polypeptide associated with cyclin D1 and CDK2, p36 bands were purified from cyclin D1 and CDK2 immunoprecipitates, separated on SDS-PAGE and their partial V8 proteolytic mapping pattern was compared with that of PCNA. Digestion of cyclin D1-associated p36 by V8 protease revealed the same pattern as that of PCNA derived from anti-PCNA immunoprecipitates and in vitro translated PCNA. Similarly, the digestion patterns of CDK2- and CDK5-associated p36 also match to that of PCNA. The p36 associated with cyclin D1 is PCNA. In addition, proteolytic mapping of the p21 seen in anti-PCNA immunoprecipitate (data not shown) showed it to be the same as cyclin D1-associated p21.

Although the experimental techniques used in this study do not formally allow a distinction between the existence of multiple pair-wise interactions between each protein, the data are most simply explained if D cyclin, PCNA, CDK and p21 form a quaternary complex, as illustrated (Figure 1B). As judged by the intensity of the methionine-labelled bands in the immunoprecipitation reactions, not all the cyclin D is present in the complex, nor is all the PCNA (data not shown). However, the relative intensity of the p36 (PCNA), p33 (CDK4) and p21 bands in an anti-cyclin D precipitate is very similar (data not shown). The results presented herein do not rule out the possibility that cyclin D, with or without the associated proteins described here, might associate with additional partners in vivo. In particular, two polypeptides that migrate either side of the 97KD molecular weight marker are apparent in anti-cyclin D precipitation reaction (data not shown).

PCNA has been described as an essential accessory factor to the delta polymerase, that is required both for leading-strand DNA replication and also for DNA repair (Prelich, G. et al., Nature, 326:517-520 (1987); Prelich G. and B. Stillman, Cell, 53:117-126 (1988); Toschi, L. and R. Bravo, J. Cell Biol.. 107:1623-1628 (1988); M.K.K. Shiviji, et al., Cell, 69:367-374 (1992). It localizes in the nucleus at sites of active DNA synthesis and the localization of PCNA, but not its synthesis, is dependent on DNA synthesis. It was not possible to detect phosphorylation of any of the respective subunits in in vitro kinase reactions, suggesting that neither PCNA nor p21 is a primary substrate of cyclin D/CDK.

The cyclin D/CDK enzymes that associate with PCNA and p21 might assemble in vivo into a more elaborate multi-protein-DNA synthetic complex, one component of which might be the physiological substrate of cyclin D/CDK.

PCNA has generally been biochemically purified from cells in a monomeric form that is unassociated with other proteins (Prelich, G. et al., Nature 346:760-763 (1987)). It is possible that the multi-protein complexes described in the present study were over-looked because they do not comprise the majority of the cellular PCNA. Alternatively, it is possible that PCNA has further non-DNA synthetic cell cycle regulatory roles that have not previously been described and that involve cyclin D and CDK proteins. However, the present studies do provide the first biochemical indication of a possible function of D-type cyclins, as modulators of PCNA function.

The importance of the quaternary complex is emphasized by the new discovery that cellular transformation by DNA tumor viruses is associated with selective subunit rearrangement of the cyclin D complexes, as well as other cell cycle complexes, including cyclin A, CDC2, CDK2, CDK4 and CDK5 complexes. In particular, introduction of SV40 DNA tumor virus or its oncogenic gene product large T antigen into normal human diploid fibroblasts (HDF) causes disruption of the association between cyclin D and PCNA, CDKs (such as CDK2, CDK4 and CDK5) and p21. After dissociation from cyclin D and p21, CDK4 kinase becomes associated with a 16 kDa polypeptide (p16). Similarly, SV40 transformation causes a decrease of association of p21 with cyclin A in HDF; and adenovirus-(293 cell line) or human papilloma virus- (HeLa cell line) transformed cells, p21 is completely disassociated from cyclin A. A 19 kDa peptide, p19, then appears in a complex with cyclin A. Therefore, p21 is associated with cyclin kinases only in normal, untransformed cells, whereas p16, p19 and possibly other related proteins are present in cyclin complexes in transformed cells.

Uses of the Invention

Based on work described herein, it is possible to detect altered complexes of cyclins CDK, PCNA and p21 in cells obtained from a tissue or biological sample, such as blood, urine, feces, mucous or saliva. This has potential for use for diagnostic and prognostic purposes since, for example, there appears to be a link between alteration of a subunit composition of composition of complexes and cellular transformation or abnormal cell proliferation. Diagnostic and therapeutic methods described herein can be used to assess an individual's disease state or probability of developing a condition associated with or caused by such rearrangements of complex subunits, and to monitor therapy effectiveness (by assessing the effect of a drug or drugs on subunit rearrangement.

For example, an agent can be developed that recognizes the interactions between CDKs, cyclins, PCNA and low molecular weight polypeptides (such as p21, p19 and p16). The agent can then be contacted with the sample of cells for which the transformation state is to be tested; presence of particular subunits in a complex will be indicative of transformation. For example, a CDK4-p16 complex will be indicative of transformation, as will a cyclin A-p19 complex. Alternatively, agents which recognize different subunits can be used in conjunction, to determine the presence of interactions among the subunits. For example, an agent which recognizes p21 can be used in conjunction with an agent which recognizes a cyclin or a cyclin kinase, to determine whether p21 is complexed with either the cyclin or the cyclin kinase.

Antibodies specifically reactive with compounds of the quaternary complexes can be produced, using known methods, to be used as agents in these methods. For example, antisera can be produced by injecting an appropriate host (e.g., rabbits, mice, rats, pigs) with the

D-type cyclin against which anti sera is desired and withdrawing blood from the host animal after sufficient time for antibodies to have been formed. Monoclonal antibodies can also be produced using known techniques. Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); Hallow, E. and D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Press, New York (1988). Antibodies specifically reactive with CDK5, can also be produced using known methods.

The present invention also includes a method of screening compounds or molecules for their ability to inhibit or suppress the transformation of a cell. A compound or molecule to be assessed for its ability to inhibit transformation is contacted with the cells, under conditions appropriate for entry of the compound or molecule into the cells. Transformation of the cell will result in selective rearrangement of subunits in the cyclin complexes. Comparison of the rate or extent of rearrangement in the presence of the compound or molecule being assessed with that of an appropriate control (e.g., the same type of cells without added test drug) will demonstrate the ability or inability of the compound or molecule to inhibit subunit rearrangement. Drugs which inhibit subunit rearrangement are also the subject of this invention.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLE 1 Demonstration that D-Type Cyclins Associated with Multiple Protein Kinases and the DNA Replication and Repair Factor PCNA

Experimental Procedures

Cells

Human diploid lung fibroblast WI38 cells were obtained from American Type Culture Collection at passage 13 and were grown in Dulbecco-Modified Eagle media supplemented with 10% fetal bovine serum and used between passages 16-22. 293 cells were cultured similarly.

Antibodies

To raise anti-cyclin D1 antibody, a 609 bp DNA restriction fragment encoding 202 amino acid residues (~25 kDa) of human cyclin D1 amino-terminal region (the NCoI fragment from nucleotides 143 to 751 in Figure 2 of Xiong, et al., Cell 65:691-699 (1991 and Current Biology 1:362-364 (1991)) was subcloned into a phage T7 expression vector, pET-3d (Studier, et al., Methods in Enzymology, 185:60-89 (1990)) and introduced into E. coli strain BL21 (DE3). Bacterial extracts were prepared in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH7.5 and 10% glycerol) by disrupting cells with sonication and clarifying the supernatant by centrifugation at 20,000 g for 10 minutes. Pellets containing insoluble cyclin D protein was resuspended in lysis buffer supplemented with 8 M urea, after 30 minutes shaking at room temperature, the suspension was centrifuged again at 20,000 g for 10 minutes. Pellets containing insoluble cyclin D protein was resuspended in SDS sample buffer and separated on 10% SDS-polyacrylamide gel. The 25 kDa cyclin D protein was visualized and excised after staining the gel with 0.25M KCl in the cold room. Gel slices were further crushed by repeated passage through an 18 gauge needle and cyclin D protein was extracted by incubating the crushed gel particles with PBS containing 0.1% SDS at 42°C for several hours and used for

injection of rabbits. To affinity purify the anti-cyclin D1 immunoglobulins, bacterially produced p25 proteins were cross-linked to the Reacti-Gel (6X) according to the manufacturer's instruction. The affinity column was washed with excess volume of PBS containing 0.05% Tween-20 before and after crude serum was applied to the column. Bound immunoglobulins were eluted with Glycine-NaCl (pH2.5) into 1.5 M Tris-HCl, pH8.5 to instantly neutralize the antibodies. To reduce the high background caused by immunoglobulin proteins, affinity purified anti-cyclin D1 was crosslinked to protein A agarose beads according to Harlow and Lane, Antibodies: a laboratory manual, Cold Spring Harbor Laboratory Press, NY (1988). On Western blots, the anti-cyclin D1 antiserum weakly cross-reacts with bacterially produced human cyclin D2, very poorly with bacterially produced human cyclin D3, and detects a single band from total WI38 cell lysates. In the immunoprecipitations with RIPA buffer (0.1% SDS), more than 90% of cyclin D1-associated p36, p33, p31 and p21 are disappeared while the amount of cyclin D1 remained to be the same as that in the immunoprecipitations with NP40 (0.5%) buffers.

For anti-CDK5 antibody production a peptide CYFSDFCPP with the underlined amino acid residues corresponding to the carboxy-terminal region of CDK5 was synthesized. The peptide was coupled to keyhole limpet hemocyanin (Pierce) which was then used to immunize rabbits as described (Green, *et al.*, Cell 28:477-487 (1982)).

Anti-cyclin D3 peptide antibody was similarly raised against a synthetic peptide CDELDQASTPTDVRDIDL with the underlined region corresponding to the carboxy-terminal region of human cyclin D3. The rabbit was later stimulated with bacterial produced full length human cyclin D3. Cyclin D3 specific immunoglobulins were purified on an affinity column in which the 17-mer cyclin D3 peptides

were crosslinked to the Reacti-Gel (6X). The affinity purified anti-cyclin D3 peptide antibody does not cross-react with bacterially produced cyclin D1 or D2 on Western blots and does not immunoprecipitate cyclin D1 from W138 cell lysates.

The antiserum against S. pombe p34^{cdc2} (G8) was described before (Draetta, et al., Cell 50:319-325 (1987)). Human auto-immune anti-PCNA antiserum was from Dr. Michael Mathews (Cold Spring Harbor Laboratory, New York). Affinity purified anti-PCNA monoclonal antibody used in Western-blots was purchased from Boehringer Mannheim. Affinity purified anti-PCNA monoclonal antibody used in immunoprecipitation of Figure 6B was purchased from Oncogene Science. Anti-CDK2 peptide antiserum was a gift of Dr. Giulio Draetta (EMBL, Heidelberg, Pagno, et al., 1992b) and does not cross-react with CDC2, CDK4 and CDK5 polypeptides. Anti-CDK4 antiserum was a gift of Dr. Steven Hanks (Vanderbilt University, Tennessee) and was raised against a fusion protein of glutathione S transferase (GST) and a C-terminal portion of CDK4. It does not cross-react with CDK2 and CDK5.

Screening Human cDNA Expression Library

A human HeLa cell cDNA expression library constructed in lambda ZAP II (#936201) was from Stratagene. Human p34^{cdc2} was highly insoluble when produced from bacteria. The conventional antibody screening method (Young and Davis, Proc. Natl. Acad. Sci. 80:1194-1198 (1983)) is suitable only when there is sufficient amount of soluble recombinant proteins in phage plaques. The screening method, therefore, was modified to include a step which involved the use of 6M guanidine to solubilize recombinant proteins after they have been transferred to nitrocellulose paper, a procedure which was initially developed to produce refolded recombinant proteins with certain activi-

ties (Vinson, et al., Gene Dev. 2:801-806 (1988)). Two million phage plaques from the λZAP II HeLa cDNA library were screened with antiserum against S. pombe $p34^{cdc2}$ (G8). After overlaying phage plaques with IPTG-impregnated nitrocellulose filters for 4 hours at 42°C, the filters were removed from culture dishes and were then treated with 6 M guanidine-HCl in a buffer containing 25 mM Hepes, pH7.0, 50 mM NaCl, 2 mM DTT for 10 min at 25°C. The filters were washed free of guanidine with Tris-buffered saline before antibody incubation. This procedure enhanced our antibody detection signal greatly which probably was due to the solubilization of bacterial-produced polypeptide precipitates by guanidine. The G8-positive cDNA clones subcloned into pBluescript SK vector (Stratagene) and sequenced from both directions using ABI automated DNA sequencer (Model 373A). For sequence homology search, the FASTA program was used (Pearson and Lipman, Proc. Natl. Acad. Sci. 85:2444-2448 (1988)).

Immunoprecipitation and Western-Blotting

For metabolic labelling with [^{35}S] methionine, subconfluent (40-60%) cells were washed twice with prewarmed labelling media (methionine-, cystine-free DMEM [ICN] supplemented with 10% dialyzed fetal bovine serum, [GIBCO]). After 30 minutes incubation with the labelling media, [^{35}S] methionine (Trans^{35}S-label, ICN) was added to media (approximately 200 μCi/ml) and continued to incubate for four to six hours before lysis. All steps of immunoprecipitations were carried out in the cold room. Cells from 40 to 60% confluent 150 mM dish were washed twice with cold PBS and scraped into NP-40 lysis buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 20 mM EDTA, 0.5% NP-40, 1mM PMSF, 25 μg/ml leupeptin, 25 μ/ml aprotitin, 1 mM benzamidine and 10 μg/ml trypsin inhibitor) and lysed by rotating for 15 to 30 minutes. Nuclei were removed by centrif-

ugation at 15,000 g for 5 minutes and lysates were pre-cleared by incubating with either pre-immune serum or normal rabbit serum and IgG sorb (The Enzyme Center, Inc.) for 20 to 30 minutes followed by a 10 minute centrifugation at 15,000 g. Antibody pre-coupled to the protein A agarose beads (Pierce) was added to the clarified lysates and incubated for six to eight hours. Immunoprecipitates were washed three to four times with lysis buffer at room temperature, resuspended in SDS sample buffer and separated on SDS-polyacrylamide gels.

For the ^{35}S methionine-labelled precipitates, polyacrylamide gels (except those for V8 proteolytic mapping experiments) were fixed with 10% glacial acetic acid and 30% methanol for 30 minutes to one hour, enhanced by impregnating with autoradiography enhances (Du Pont) for 30 minutes and precipitated in water for 15 to 30 minutes. Enhanced gels were dried and exposed to X-ray films at -70°C. For Western-blotting, polypeptides were transferred to a nitrocellulose filer using a SDE Electroblotting System (Millipore) for 45 minutes at constant current of 400 mA. The filter was blocked for 1 to 3 hours with TBST (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween-20) containing 5% dry milk, incubated with primary antibody for 4 hours to overnight in TBST containing 5% dry milk and washed 4 times, 10 minutes each time, with TBST. Appropriate secondary antibody (1:10,000 dilution of either horseradish peroxidase linked sheet anti-mouse Ig or donkey anti-rabbit Ig, Amersham) were incubated with filters for one hour and specific proteins were detected using an enhanced chemiluminescence system (ECL, Amersham).

<u>Partial Proteolytic Peptide Mapping</u>

Human cyclin D1, cyclin D2, cyclin D3, CDC2, CDK2, CDK3, CDK4, CDK5 and PCNA were subcloned into pBluescript

vector (Stratagene) for *in vitro* translation with T7 RNA polymerase using a TNT coupled reticulocyte lysate system (Promega). Immunoprecipitation of [^{35}S] methionine-labelled lysates and SDS-polyacrylamide gel electrophoresis were the same as described above. Polyacrylamide gels were dried without prior fixation and enhanced treatment, exposed to Fuji image plates and visualized on Fuji bio-imaging analyzer BAS2000. Appropriate protein bands were excised from the gels using image printout as template, in-gel partially digested with various amount of S. aureus V8 protease according to (Cleveland, *et al.*, *J. Biol. Chem.* *252*:1102-1106 (1977)) and (Harlow and Lane, *Antibodies: a laboratory manual*, Cold Spring Harbor Laboratory Press, NY (1988)), separated on a 17.5% SDS-PAGE. Gels were dried and exposed to a X-ray film for 2 weeks, or analyzed on a Fuji image analyzer BAS2000.

EXAMPLE 2 Demonstration of Selective Subunit Rearrangement of Cell Cycle Complexes In Association With Cellular Transformation by a DNA Tumor Virus or Its Oncogenic Product

Cellular Transformation With DNA Tumor Virus SV40 Is Associated With Subunits Rearrangement of Cell Cycle Complexes

Preparation of [^{35}S] methionine-labelled cell lysates and polyacrylamide gel electrophoresis were as described in the co-pending U.S. applications cited herein. Cell lysates were prepared from either human normal diploid fibroblast cells WI38 (Figure 2, lanes 1, 4, 5, 7, 8, 11, and 13) or DNA tumor virus SV40 transformed WI38 cells, VA13 (Figure 2, lanes 2, 6, 9, 10, 12 and 14). Cell lysates were immunoprecipitated with antibodies against each cell cycle gene products, as indicated at the top of

each panel. In case of immunoprecipitation with anti-CDK2 (Figure 2, lane 5) and CDK4 (Figure 2, lane 8 and 10), competing peptides were preincubated with antisera. Identified polypeptides in each immunoprecipitates were indicated at the left side of each panel and molecular weight markers were at right.

Subunit Rearrangements of Cell Cycle Complexes In Two Different Pair Cell Lines

Methods for preparation of cell lysates, gel electrophoresis as shown in Figure 3 are the same as described above. Two different pair cell lines were used in these experiments. HSF43 is a normal human diploid fibroblast cell line and CT10 (full name CT10-2C-T1) is a derivative of HSF43 transformed by SV40 large tumor antigen. CV-1 is an African green monkey kidney cell line and COS-1 is a derivative of CV-1 transformed by SV40.

Cellular Transformation by DNA Tumor Virus SV40 Is Associated With Rearrangement of PCNA Subunit of Cell Cycle Complexes

Preparation of cell lysate, electrophoresis, and Western blotting conditions are the same as described above. Normal human diploid fibroblast cell lines and their SV40 transformed cell lines are described above. Immunoprecipitates derived from each antibody as indicated at the top of each panel in Figure 4 were separated on polyacrylamide gels and blotted with anti-PCNA antibody.

Cellular Transformation by DNA Tumor Virus SV40 Is Associated With Rearrangement of CDK4 Subunit of Cell Cycle Complexes

Preparation of cell lysate, electrophoresis, and Western blotting conditions are the same as previously described. Normal human diploid fibroblast cell lines and their SV40 transformed cell lines are described above. Immunoprecipitates derived from each antibody as indicated

at the top of each panel in Figure 5 were separated on polyacrylamide gels and blotted with anti-CDK4 antibody.

Cellular Transformation by Other DNA Tumor Viruses Is Also Associated With Subunits Rearrangement of Cell Cycle Complexes

Molecular weight markers are listed at the right side and antibodies used in each immunoprecipitation are indicated at the top of Figure 6. Two additional normal human diploid fibroblast cells lines, Detriot 551 and IMR-90 show the same subunits composition as WI38, HSF43 and CV-1 as described in Figures 2 and 3. HeLa is a human cervix epitheloid carcinoma cell line containing papilloma viruses, and 293 is human embryonal kidney cell line transformed by adenovirus type 5.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method of diagnosing transformation of a cell, comprising determining whether p21 is:
   a) complexed with a cyclin kinase, a cyclin, or both, or
   b) not complexed with a cyclin kinase, a cyclin, or both,

   wherein if p21 is not complexed with a cyclin kinase, a cyclin, or both, it is indicative of transformation of the cell.

2. A method of Claim 1, wherein an antibody is used to determine whether or not p21 is complexed with a cyclin kinase, a cyclin, or both.

3. A method of Claim 1, wherein the cyclin is a D-type cyclin or an A-type cyclin and the cyclin kinase is CDK4.

4. A method of diagnosing transformation of a cell, comprising determining whether p16 is
   a) complexed with a cyclin kinase, or
   b) not complexed with a cyclin kinase,

   wherein if p16 is complexed with a cyclin kinase, it is indicative of transformation of the cell.

5. A method of Claim 4, wherein an antibody is used to determine whether or not p16 is complexed with a cyclin kinase.

6. A method of Claim 4, wherein the cyclin kinase is CDK4.

7. A method of diagnosing transformation of a cell, comprising determining whether p19 is

a) complexed with a cyclin, or

b) not complexed with a cyclin, wherein is p19 is complexed with a cyclin, it is indicative of transformation of the cell.

8. A method of Claim 7, wherein an antibody is used to determine whether or not p19 is complexed with a cyclin.

9. A method of Claim 7, wherein the cyclin is cyclin A.

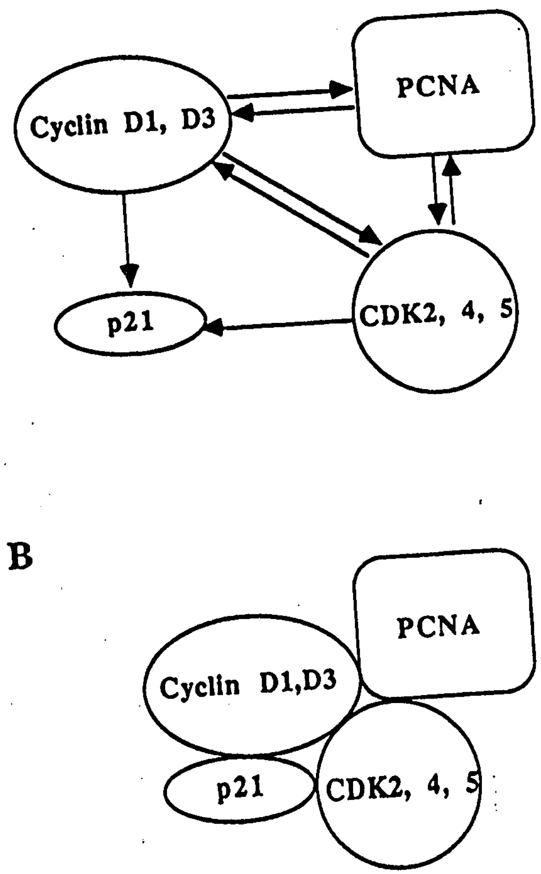
CYCLIN COMPLEX REARRANGEMENT AND USES RELATED THERETO

Abstract of the Disclosure

A method of diagnosing transformation of a cell, involving detection of the subunit components of cyclin complexes, is disclosed. In particular, the method pertains to the interaction of cyclins, PCNA, CDKs, and low molecular weight polypeptides such as p21, p19 and p16.

FIGURE 1

A



B

FIGURE 2

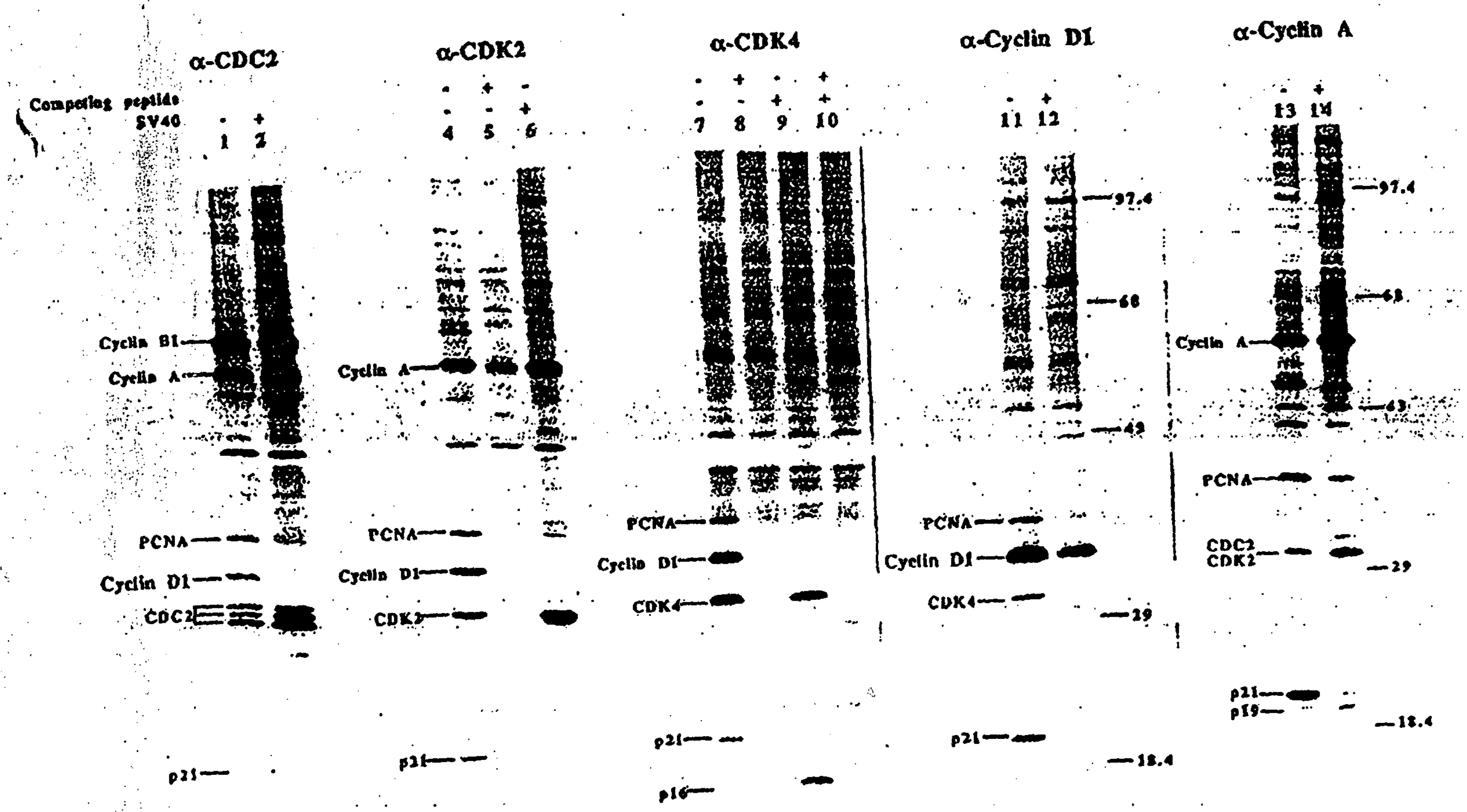
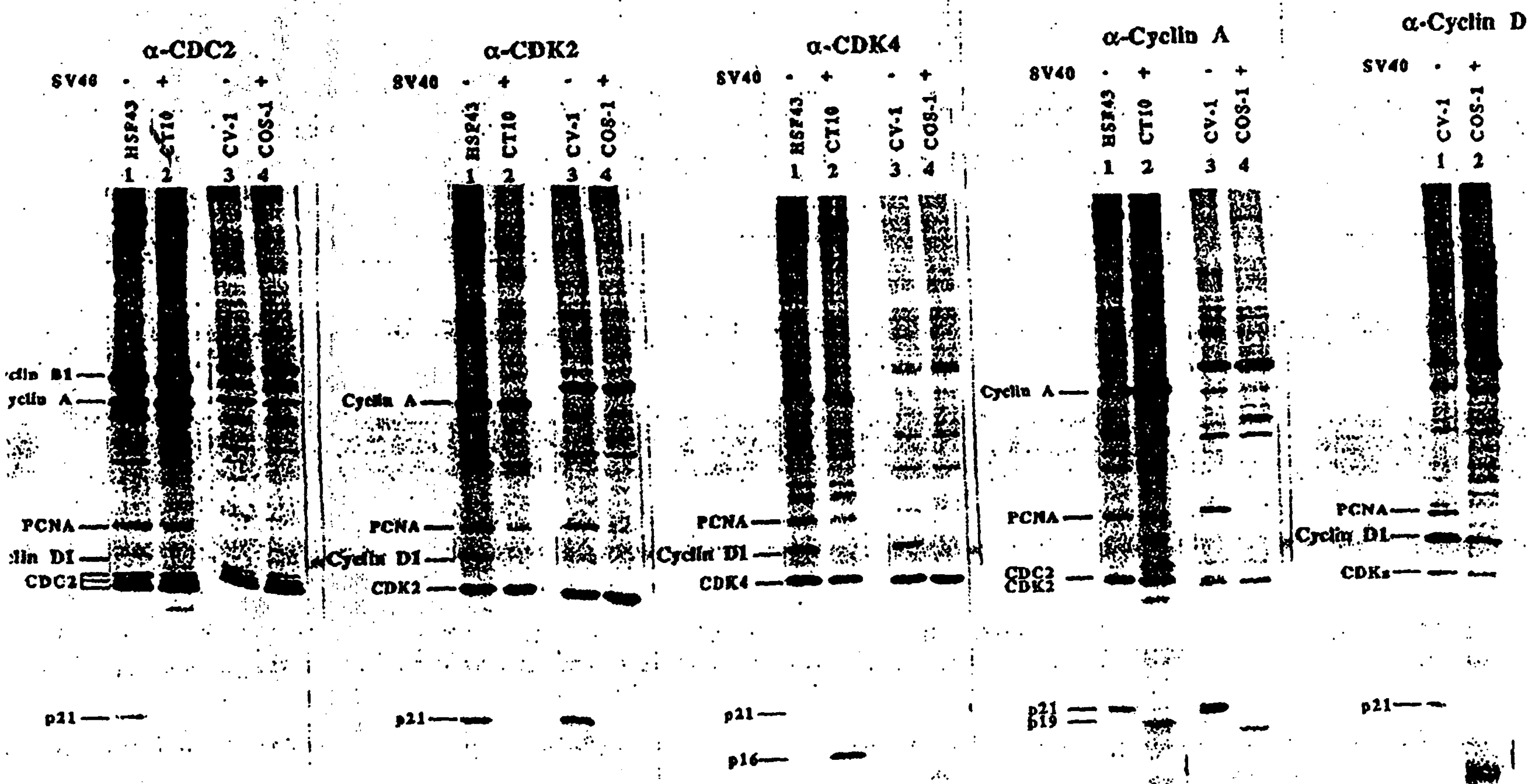

α-CDC2
α-CDK2
α-CDK4
α-Cyclin D1
α-Cyclin A
Competing peptide
SV40
1 2 4 5 6 7 8 9 10 11 12 13 14
Cyclin B1
Cyclin A
PCNA
Cyclin D1
CDC2
CDK2
CDK4
p21
p16
p19
97.4
68
43
29
18.4

FIGURE 3



α-CDC2
α-CDK2
α-CDK4
α-Cyclin A
α-Cyclin D
SV40
HSF43
CT10
CV-1
COS-1
PCNA
CDC2
CDK2
CDK4
CDKs
Cyclin A
Cyclin D1
p21
p16
p19

FIGURE 4

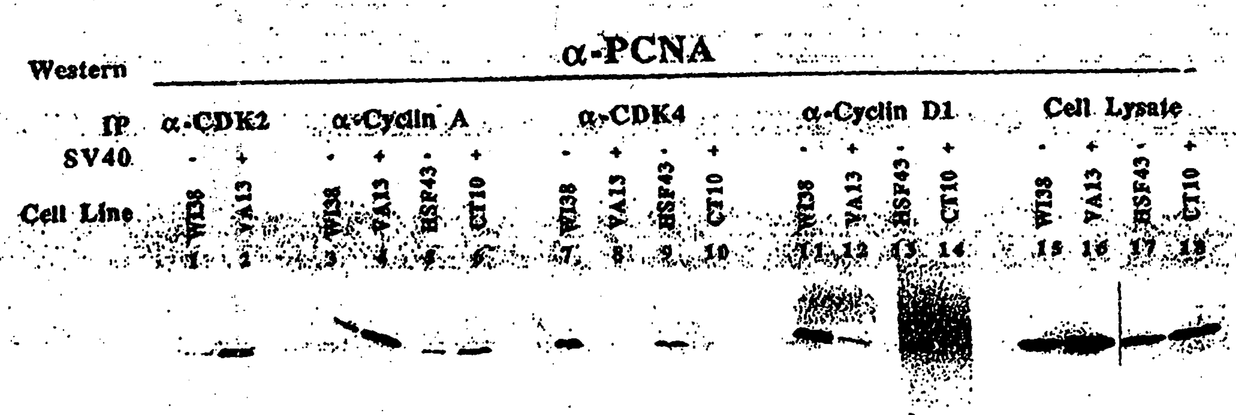
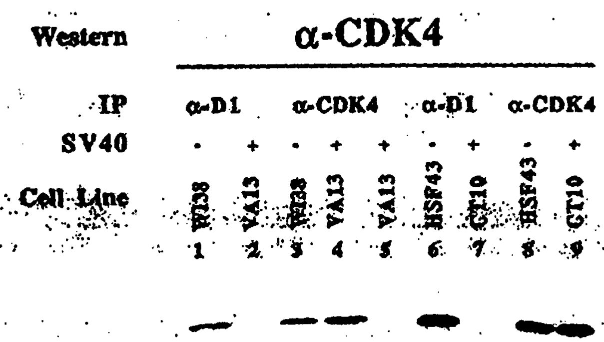

Western
α-PCNA
IP
α-CDK2
α-Cyclin A
α-CDK4
α-Cyclin D1
Cell Lysate
SV40
Cell Line
WI38
VA13
HSF43
CT10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

FIGURE 5


Western
α-CDK4
IP
α-D1
α-CDK4
α-D1
α-CDK4
SV40
- + - + + - + - +
Cell Line
WI38
VA13
WI38
VA13
VA13
HSF43
CT10
HSF43
CT10
1 2 3 4 5 6 7 8 9

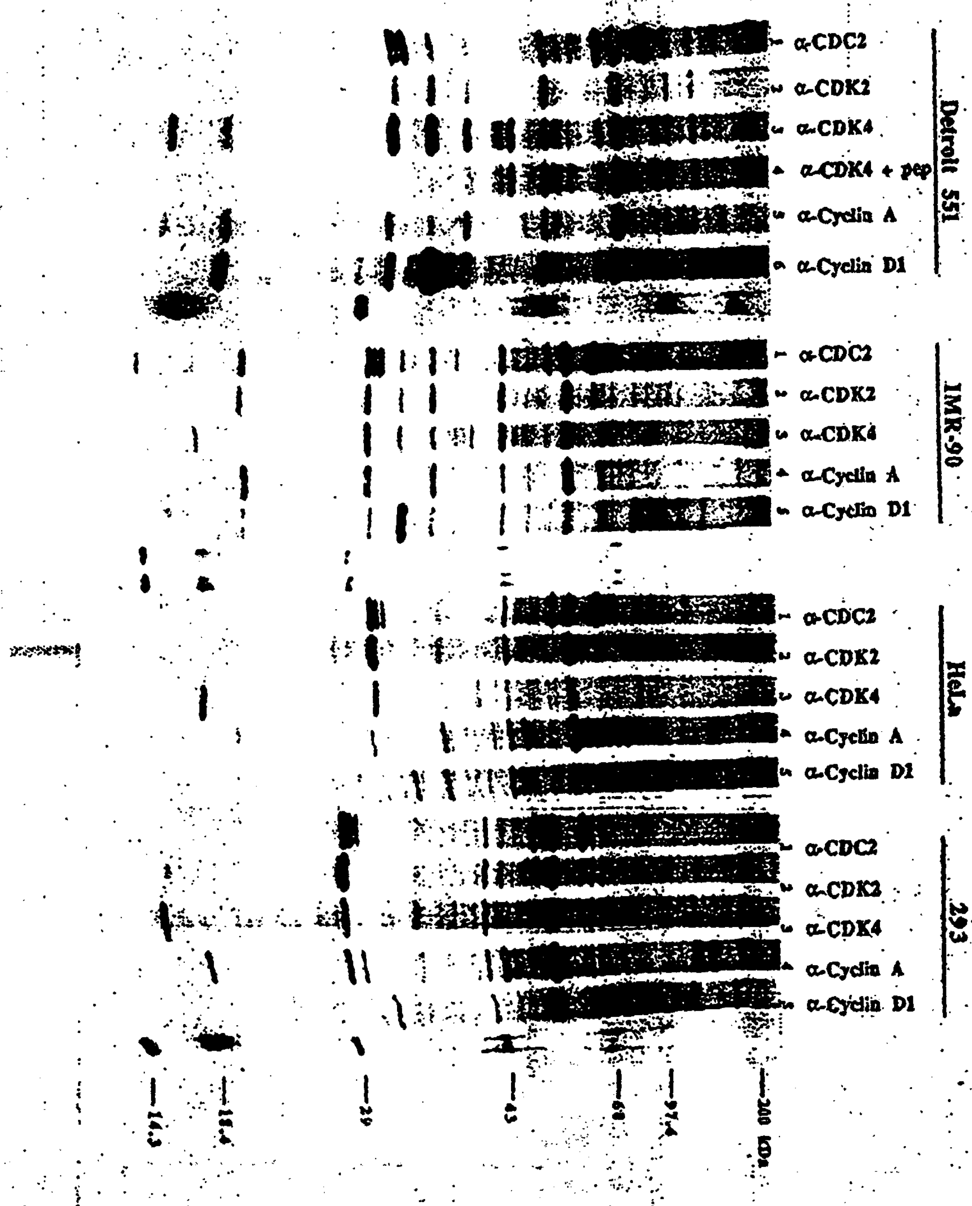

α-CDC2
α-CDK2
α-CDK4
α-CDK4 + pep
α-Cyclin A
α-Cyclin D1
Detroit 551
α-CDC2
α-CDK2
α-CDK4
α-Cyclin A
α-Cyclin D1
IMR-90
α-CDC2
α-CDK2
α-CDK4
α-Cyclin A
α-Cyclin D1
HeLa
α-CDC2
α-CDK2
α-CDK4
α-Cyclin A
α-Cyclin D1
293
14.3
18.4
29
43
68
97.4
200 kDa

FIGURE 6